Transtek Associates, Inc.

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Double-stranded ribonucleic acid with improved efficacy

The invention relates to a double-stranded ribonucleic acid (dsRNA) with a strand complementary at least in sections to a target gene, a method for preparing it, a use of this dsRNA, and a drug to inhibit the expression of a target gene.

Antisense oligonucleotides whose conjugation with lipophilic molecules improves the cellular association of antisense oligonucleotides are known from Manoharan, M., Antisense and Nucleic Acid Drug Development 2002, 12, pages 103 to 128. Cellular association leads to endocytosis in which the received antisense oligonucleotides reach the cytoplasm through endosomes and lysosomes.

It is known from Bijsterbosch, M. K. et al., Nucleic Acids Research (2000), Vol. 28, No. 14, pages 2717 to 2725, how to improve the efficacy of phosphorothioate antisense oligodesoxynucleotides by conjugation with cholesterol. Interactions with receptors and plasma proteins are held responsible for the improved antisense activity.

The fact that inhibition of gene expression by means of antisense oligonucleotides or antisense oligodesoxynucleotides is not particularly efficient is a drawback to procedures that depend on antisense technology.

Inhibition of the expression of a target gene by RNA interference is more effective. RNA interference means the targeted inhibition of the expression of a gene by double-stranded RNA (dsRNA). A dsRNA suitable for this with a strand complementary at least in sections to the target gene is disclosed in WO 00/44895. A method for inhibiting the expression of a target gene in a cell is also described in WO 00/44895, in which the dsRNA is inserted into the cell.

The objective of the present invention is to make available a dsRNA that has even better efficacy. A method for preparing such a dsRNA, its use, and a drug containing this dsRNA are also to be made available.

This objective is reached by the features of Claims 1, 11, 16, and 20. Useful refinements are found in the features of Claims 2 to 10, 11, 12 to 15, 17 to 19, and 21 to 23.

According to the invention, a double-stranded ribonucleic acid (dsRNA) is provided with a strand S1 that is complementary at least in sections to a target gene, with at least one lipophilic group being bonded to the dsRNA. The lipophilic group can be of aromatic, aliphatic, or alicyclic nature. It can be covalently bonded to the dsRNA. A dsRNA exists when the ribonucleic acid consisting of one or two ribonucleic acid strands has a double-stranded structure. Not all nucleotides of the dsRNA have to have Watson-Crick base pairs. In particular, individual base pairs not complementary to one another impair the efficacy of the dsRNA very

little or not at all. The dsRNA can have another strand S2 complementary at least to a great extent to the strand S1. The maximum possible number of base pairs is the number of nucleotides in the shortest strand contained in the dsRNA, if the dsRNA consists of two strands. "Target gene" in general means the section of a DNA strand of the double-stranded DNA in the cell that is complementary to a section of the other DNA strand of the double-stranded DNA including all transcribed regions that serves as a matrix in the transcription of the target gene. The target gene in general, therefore, is the sense strand. The strand S1 can thus be complementary to an RNA transcript or its processing product, for example an mRNA, formed in the expression of the target gene. In the case of a (+)-strand RNA virus, for example such as the Hepatitis C virus (HCV), the target gene is a portion of the coding RNA strand of the virus.

Surprisingly, it has been found that the efficiency of RNA interference provided by dsRNA is increased by conjugation of the dsRNA with a lipophilic group. This is astonishing because the intracellular mechanism underlying RNA interference is completely different from what underlies inhibition of expression by the antisense principle. It therefore cannot be assumed that the plasma proteins that interact with the dsRNA pursuant to the inventions are the same as those that also interact with cholesterol-conjugated phosphorothioate antisense oligodesoxynucleotides.

The improved efficacy of antisense oligonucleotides conjugated with lipophilic molecules or of cholesterol-conjugated phosphorothioate oligodesoxynucleotides may be based on their improved uptake in cells by receptors or cellular association. Although the dsRNA pursuant to the invention can be taken up even without an added transfection aid, the mechanism underlying the improved efficacy must be a different one.

Specifically, it has been found that the dsRNA pursuant to the invention also has improved efficacy when it is incorporated into cells by means of a transfection aid such as FuGENE 6 Transfection Reagent (Roche Diagnostics GmbH, Sandhofer Str. 116, D-68305 Mannheim, Germany). Improved uptake in the cells by cellular association or receptors cannot be responsible for this.

The lipophilic group is preferably bonded, in particular covalently bonded, only to the S1 strand or only to the other strand S2 of the dsRNA that is at least to a great extent complementary to it. Astonishingly, the strand of the dsRNA that has no lipophilic group is taken up well by cells along with the strand of the dsRNA that has the lipophilic group.

It has proved to be advantageous for the lipophilic group to be bonded, in particular covalently bonded, to the 5' end of the strand S1 and/or of the strand S2. Such a dsRNA is easily prepared by solid phase synthesis and has good efficacy. The activity of such a dsRNA can be further increased by bonding the lipophilic group exclusively to the 5' end of the S2 strand.

The dsRNA shows good efficacy if the lipophilic group has the structure of a steroid, particularly of a sterol, preferably of cholesterol or of an aliphatic hydrocarbon, especially a branched hydrocarbon. The lipophilic group can have the structure of (6-hydroxyhexyl)carbamic acid cholesteryl ester or of 12-hydroxydodecanoic acid bisdecylamide.

The efficacy of the dsRNA can be increased even further if it has at its end that has the 3' end of the S1 strand, a single-stranded overhang on the S1 strand formed of 1 to 4, especially 1 or 2, nucleotides. The other end of the dsRNA can be made with no overhang, in other words smooth. An overhang at one end of the dsRNA is sufficient to intensify the interference activity. This does not impair the stability of the dsRNA to such a degree as two overhangs. A dsRNA with only one overhang has proved to be adequately resistant and particularly active both in various cell culture media and in blood, serum, and cells.

It is preferred for the S1 strand of the dsRNA to have fewer than 30, particularly fewer than 25, nucleotides. The number of nucleotides in the S1 strand should be at least 17. The S1 strand preferably has 21 to 24 nucleotides. Such a dsRNA also called siRNA (short interfering RNA) for brevity, has proved to show particular intracellular stability. The S2 strand preferably has the same number of nucleotides at the most. The target gene can be a target gene expressed, particularly specifically, in liver cells or pancreatic cells, in the uterus, or in the urinary bladder. In the case of liver cells, they are especially endothelial cells, Kupffer's cells, or parenchymal cells. The dsRNA pursuant to the invention is especially active with such liver cells because it is absorbed especially efficiently by these cells. This is particularly true when the lipophilic group has a cholesterol framework. However, good uptake of the dsRNA also occurs with cells from other tissues such as the pancreas, urinary bladder, and uterus, especially the cervix. The dsRNA pursuant to the invention is especially well suited for treating an infection with the HCV virus involving liver cells. For this, the target gene originates from the HCV genome, especially the untranslated region at the 3' end of the HCV genome (3' UTR).

The invention also relates to a method for preparing a dsRNA pursuant to the invention, wherein the RNA constituting the S1 strand and/or the S2 strand is prepared by solid phase synthesis from the 3' end to the 5' end, and a lipophilic molecule that has a phosphoramidite group is coupled to the 5' end of the RNA in the last cycle of synthesis. The nucleotides at first exist as nucleoside phosphoramidites in the solid phase synthesis of an RNA occurring in successive synthetic cycles. An additional nucleoside phosphoramidite is bonded to the 5' OH group of the nucleotide last incorporated in each synthetic cycle. If the lipophilic molecule has a phosphoramidite group, it can be coupled like a nucleoside phosphoramidite to the free 5' OH end of the previously synthesized RNA in the solid phase synthesis. This distinctly simplifies the synthesis of a dsRNA containing a lipophilic group. The synthesis can be automated and standardized to be carried out in a conventional RNA synthesizer. The synthesis of the lipophilic molecule having the phosphoramidite group can include the phosphitylation of a free OH group to give the phosphoramidite group. The phosphoramidite group can thereby be made available

particularly easily. The synthesis of the lipophilic molecule having the phosphoramidite group can include conversion of cholesteryl chloroformate into an acid amide, or reaction of 12-hydroxylauric acid with di(n-decyl)amine to form an acid amide bond. The lipophilic molecule that has the phosphoramidite group can be [6-(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]carbamic acid cholesteryl ester, or 12-[(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]dodecanoic acid bis(decyl)amide.

The invention also relates to the use of a dsRNA pursuant to the invention to inhibit the expression of a target gene contained in cells. This can occur in vitro or in vivo. The use is especially beneficial when no agent is used by which the absorption of the dsRNA is facilitated. Side effects caused by such an agent can thereby be avoided. An example of such an agent is a transfection aid such as FuGENE 6 Transfection Reagent, which is sold by the company Roche Diagnostics GmbH. However, this agent cannot be used in vivo. The expression of the target gene in liver cells or pancreatic cells or in cells of the uterus or of the urinary bladder, especially those infected with the HCV virus, is inhibited. The target gene can originate from the HCV genome, particularly from the untranslated region located at the 3' end of the HCV genome (3'-UTR).

The invention also relates to a drug containing the dsRNA pursuant to the invention for inhibiting the expression of a target gene. The drug preferably contains no agent by which the absorption of dsRNA in the cells is facilitated. The cells can be liver cells or cells from the pancreas, uterus, or urinary bladder, especially those infected with the HCV virus. The target gene can originate from the HCV genome.

The invention will be described below with reference to the drawings by way of examples. The drawings show

Fig. 1 a schematic illustration of a synthetic route for preparing "Chol",

Fig. 2 a schematic illustration of a synthetic route for preparing "C32",

Fig. 3 the relative β-galactosidase activity in β-Gal⊕HuH-7 cells after transfection with dsRNA,

Fig. 4 the relative β-galactosidase activity in β-Gal⊕HuH-7 cells after transfection with dsRNA without using a transfection aid, and a Northern Blot of the dsRNA isolated from the cells after transfection, and

Figs. 5a, b, c each shows a Northern Blot of dsRNA that was isolated from the particular cells after transfection of cells of the pancreas, uterus, and urinary bladder without using a transfection aid.

Synthesis of the cholesterol derivative "Chol":

The synthetic route for preparing Chol is shown schematically in Fig. 1.

For the synthesis, cholesteryl chloroformate 8 was converted as follows to the acid amide (6-hydroxyhexyl)carbamic acid cholesteryl ester 9 by adding 1.2 equivalents of 6-aminohexanol in the presence of $NaHCO_3$:

2 g (4.45 mol) of cholesteryl chloroformate 8 was dissolved in 10 ml of CH_2Cl_2, and 561 mg (6.68 mmol) of $NaHCO_3$ was added. 626 mg (5.34 mmol) of 6-aminohexanol dissolved in 10 ml of CH_2Cl_2 was then added with vigorous stirring at 0 °C. The addition rate was about 1 drop/s. The reaction ran for 16 h, with the temperature slowly rising to room temperature. The reaction mixture was then diluted with 30 ml of CH_2Cl_2 and extracted with H_2O, and the aqueous phase was back-extracted with CH_2Cl_2. The combined organic phases were then dried over Na_2SO_4, filtered, and evaporated. The crude product thus obtained was purified by column chromatography. A column 4.5 cm in diameter and 15 cm tall packed with silica gel, particle size 40-63 µm (Merck KgaA, Frankfurter Str. 250, Darmstadt, Germany) was used for this. The mobile phase used was 2:1 cyclohexane/ethyl acetate.

The free OH group of the resulting product 9 was phosphitylated as follows, i.e. converted into a phosphoramidite group:

500 mg (0.944 mmol) of the educt 9 was dissolved in 15 ml of anhydrous CH_2Cl_2 and was placed in a countercurrent of argon in a flask that was previously evacuated and ventilated with argon. 485 µl (2.83 mmol) of N,N-diisopropylethylamine (DIPEA) was added with stirring, 261 µl (1.04 mmol) of 2-cyanoethyldiisopropylchlorophosphoramidite was then added dropwise, and the reaction mixture was stirred in a stream of argon at room temperature. After 90 minutes the reaction was completed. The reaction mixture was first diluted with CH_2Cl_2 and then extracted with saturated NaCl solution. Finally, the aqueous phase was back-extracted with CH_2Cl_2. The combined organic phases were then dried over Na_2SO_4, filtered, and evaporated in a rotary evaporator ventilated with argon. The crude product was purified by column chromatography with argon pressure. A column 4.5 cm in diameter and 8 cm tall packed with silica gel, particle size 40-63 µm (Merck KGaA) was used for this. 6:1 cyclohexane/ethyl acetate + 1 % triethylamine (Et_3N) was used as the mobile phase. The silica gel used was previously slurried for 2 hours with cyclohexane/1 % Et_3N. The [6-(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]carbamic acid cholesteryl ester 10, called "Chol", was obtained as the product.

Synthesis of the di-n-decylamine derivative "C32":

The synthetic route for preparing C32 is shown schematically in Fig. 2.

For the synthesis, 12-hydroxylauric acid 11 was reacted as follows with 2 equivalents of the secondary amine di-n-decylamine 15 to give the product 12-hydroxydodecanoic acid bisdecylamide 16:

1 g (4.623 mmol) of 12-hyudroxylauric acid 11 was caused to react with 282 mg (2.31 mmol) of p-N,N-dimethylaminopyridine (DMAP) and 1.329 g (6.94 mmol) of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI). 2.751 g (9.246 mmol) of di-n-decylamine 15 was then added and the reaction mixture was stirred vigorously at room temperature. After 16 hours the crude product was diluted with CH_2Cl_2 and extracted twice with H_2O, with a flocculent white solid being formed in the aqueous phase. The aqueous phase was back-extracted with CH_2Cl_2. The organic phases were then dried over Na_2SO_4, filtered, and evaporated. The crude product obtained in this way was purified by column chromatography. A column 4.5 cm in diameter and 19 cm tall packed with silica gel, particle size 40-63 µm (Merck KgaA) was used for this. 4:1 cyclohexane/ethyl acetate was used as the mobile phase.

The free OH group of the resulting product 16 was phosphitylated as follows:

1.018 g (2.05 mmol) of the acid amide 16 was dissolved in 30 ml of anhydrous CH_2Cl_2 and placed in a countercurrent of argon in a flask that was previously evacuated and ventilated with argon. 1054 µl (6.16 mmol) of DIPEA was then added with stirring, 504 µl (2.26 mmol) of 2-cyanoethyldiisopropylchlorophosphoramidite was added dropwise, and the reaction mixture was stirred in a stream of argon at room temperature. After 2 hours the reaction was completed. The reaction mixture was first diluted with CH_2Cl_2, and then extracted with saturated NaCl solution. Finally, the aqueous phase was back-extracted with CH_2Cl_2. The combined organic phases were then dried over Na_2SO_4, filtered, and evaporated in a rotary evaporator ventilated with argon. The crude product was purified by column chromatography under argon pressure. A column 4.5 cm in diameter and 20 cm tall packed with silica gel, particle size 40-63 µm (Merck KgaA) was used for this. The mobile phase used was 8:1 cyclohexane/ethyl acetate + 1 % Et_3N. The silica gel used was previously slurried for 2 hours with cyclohexane/1 % Et_3N. The 12-[(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]dodecanoic acid bis(decyl)amide 17, called "C32" here, was obtained as the product.

The chemicals used for the synthesis of Chol and C32 originated from Fluka, Industriestrasse 25, CH-9471 Buchs SG, Switzerland, and the constituents of the mobile phase used for the column chromatograph were obtained from Carl Roth GmbH & Co., Schoemperlenstr. 1-5, 76185 Karlsruhe, Germany.

RNA synthesis:

A sequence from the highly preserved 3'-UTR region of the Hepatitis C virus (HCV) gene and a sequence from the β-galactosidase (β-Gal) gene from *E. coli* were chosen as the target sequences

for RNA interference. The double-stranded oligoribonucleotides used for transfections have the following sequences, designated No.1 to No. 4 in the sequence protocol:

HCV: dsRNA, one strand of which S1 (antisense strand) is complementary to a target sequence from the HCV gene:

S2: 5′- ACG GCU AGC UGU GAA AGG UCC- 3′ (HCV11s - Sequence No. 2)
S1: 3′-AG UGC CGA UCG ACA CUU UCC AGG- 5′ (HCV2 - Sequence No. 1)

Gal: dsRNA, one strand of which S1 (antisense strand) is complementary to a target sequence from the β-galactosidase gene:

S2: 5'- GUG AAA UUA UCG AUG AGC GUG- 3' (Gal3s - Sequence No. 4)
S1: 3'-GC CAC UUU AAU AGC UAC UCG CAC- 5' (Gal2 - Sequence No. 3)

A dsRNA called K22 was used as the negative control, which has no relationship to a gene expressed here. The sequences of the two oligoribonucleotides forming the K22 dsRNA are contained in the sequence protocol as sequences No. 5 and 6.

The single RNA strands were prepared by means of an RNA synthesizer (Model Expedite 8909, Applied Biosystems, Weiterstadt, Germany) and conventional solid phase synthesis using ribonucleoside phosphoramidites (ChemGenes Corporation, Ashland Technology Center, 200 Homer Avenue, Ashland, MA 01721, USA). A lipophilic group could be bonded to each of the single RNA strands. To do this, similarly to the ribonucleoside phosphoramidites, each of the amidites [6-(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]carbamic acid cholesteryl ester 10 or 12-[(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]dodecanoic acid bis(decyl)amide 17 was coupled to the 5' OH end of the synthesized RNA.

RNA single strands with and without lipophilic groups were purified by HPLC. NucleoPac PA-100, 9x250 mm columns from Dionex GmbH, Am Wortzgarten 10, 65510, Idstein, Germany, were used as columns; low-salt buffer used: 20 mM Tris, 10 mM $NaClO_4$, pH 6.8, 6 M urea; high-salt buffer used 20 mM Tris, 400 mM $NaClO_4$, pH 6.8, 6 M urea. The flow rate was 3 ml/minute. The hybridization of the single strands to form a double strand was performed by heating the stoichiometric mixture of single strands in 10 mM sodium phosphate buffer, pH 6.8, 100 mM NaCl, at 80-90 °C, and then slowly cooling to room temperature over a period of 6 hours.

The following dsRNAs and siRNAs modified with lipophilic groups were synthesized:

Gal with the lipophilic group Chol on the S1 strand: GalChol-as

Gal with the lipophilic group Chol on the S2 strand: GalChol-s

Gal with the lipophilic group Chol on both the S1 strand and the S2 strand: GalChol-2

Gal with the lipophilic group C32 on the S1 strand: GalC32-as

Gal with the lipophilic group C32 on the S2 strand: GalC32-s

Gal with the lipophilic group C32 on both the S1 strand and the S2 strand: GalC32-2

HCV with the lipophilic group Chol on the S1 strand: HCVChol-as

HCV with the lipophilic group Chol on the S2 strand: HCVChol-s

HCV with the lipophilic group Chol on both the S1 strand and the S2 strand: HCVChol-2

HCV with the lipophilic group C32 on the S1 strand: HCVC32-as

HCV with the lipophilic group C32 on the S2 strand: HCVC32-s

HCV with the lipophilic group C32 on both the S1 strand and the S2 strand: HCVC32-2

The effect of RNA interference on viral gene sequences was studied on a non-pathogenic substitute system. To do this, an oligonucleotide according to sequence No. 7 of the sequence protocol was cloned in front of a gene coding for E. coli β-galactosidase from the commercially available expression vector pβGal-Control (BD Biosciences Clontech, Tullastrasse 4, D-69126 Heidelberg, Germany, Gene Accession Number U13186; nucleotide 280-3429). Sequence No. 7 corresponds to a sequence comprising 24 nucleotides from a highly preserved region of the 3'-UTR of the HCV genome. The fusion genes generated in this way were cloned into the commercially available expression plasmid pcDNA3.1 (+) (Invitrogen, Life Technologies, Technologiepark Karlsruhe, Emmy-Noether-Str. 10, D-76131 Karlsruhe, Germany, Catalog Number V790-20), which contains a resistance gene against the antibiotic neomycin. The resulting plasmid was called p3. In p3, the HCV sequence conforming to sequence No. 7 is not part of the open reading frame of the sequence coding for β-galactosidase, so that the HCV sequence is actually expressed as part of an mRNA coding for β-galactosidase but not as part of a fusion protein. The sequence of the mRNA corresponding to the 24 nucleotides is then identical with the corresponding sequence of the HCV genome. The plasmid p3 has the following relevant sequence section according to sequence No. 8 of the sequence protocol:

GTC ACC TTG TCG TCA CGG CTA GCT GTG AAA GGT CCA GTC ACC ATG TCG TTT ACT TTG
M S F T L

Gal with the lipophilic group Chol on the S2 strand: GalChol-s

Gal with the lipophilic group Chol on both the S1 strand and the S2 strand: GalChol-2

Gal with the lipophilic group C32 on the S1 strand: GalC32-as

Gal with the lipophilic group C32 on the S2 strand: GalC32-s

Gal with the lipophilic group C32 on both the S1 strand and the S2 strand: GalC32-2

HCV with the lipophilic group Chol on the S1 strand: HCVChol-as

HCV with the lipophilic group Chol on the S2 strand: HCVChol-s

HCV with the lipophilic group Chol on both the S1 strand and the S2 strand: HCVChol-2

HCV with the lipophilic group C32 on the S1 strand: HCVC32-as

HCV with the lipophilic group C32 on the S2 strand: HCVC32-s

HCV with the lipophilic group C32 on both the S1 strand and the S2 strand: HCVC32-2

The effect of RNA interference on viral gene sequences was studied on a non-pathogenic substitute system. To do this, an oligonucleotide according to sequence No. 7 of the sequence protocol was cloned in front of a gene coding for E. coli β-galactosidase from the commercially available expression vector pβGal-Control (BD Biosciences Clontech, Tullastrasse 4, D-69126 Heidelberg, Germany, Gene Accession Number U13186; nucleotide 280-3429). Sequence No. 7 corresponds to a sequence comprising 24 nucleotides from a highly preserved region of the 3'-UTR of the HCV genome. The fusion genes generated in this way were cloned into the commercially available expression plasmid pcDNA3.1 (+) (Invitrogen, Life Technologies, Technologiepark Karlsruhe, Emmy-Noether-Str. 10, D-76131 Karlsruhe, Germany, Catalog Number V790-20), which contains a resistance gene against the antibiotic neomycin. The resulting plasmid was called p3. In p3, the HCV sequence conforming to sequence No. 7 is not part of the open reading frame of the sequence coding for β-galactosidase, so that the HCV sequence is actually expressed as part of an mRNA coding for β-galactosidase but not as part of a fusion protein. The sequence of the mRNA corresponding to the 24 nucleotides is then identical with the corresponding sequence of the HCV genome. The plasmid p3 has the following relevant sequence section according to sequence No. 8 of the sequence protocol:

GTC ACC TTG TCG TCA CGG CTA GCT GTG AAA GGT CCA GTC ACC ATG TCG TTT ACT TTG
M S F T L

The N-terminal amino acid sequence of the fusion product HCV β-galactosidase is presented below the DNA sequence. The HCV sequence is shown italicized. The beginning of the β-Gal gene (including 6 nucleotides of the Kozak sequence before the codon ATG) is underlined.

All experiments to study the post-transcriptional inhibition of gene expression by RNA interference were carried out with cells of the β-Gal⊕HuH-7 cell line derived from HuH-7 cells (JCRB0403, Japanese Collection of Research Bioresources Cell Bank, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158, Japan). HuH-7 is a human hepatoma cell line. The HuH-7 cells were transfected with p3. The transfected HuH-7 cells have resistance to the neomycin analog G418, so that in the presence of G418 only HuH-7 cells that have accepted the plasmid gene and with it the reporter gene in their genome are selected. These cells are called β-Gal⊕HuH-7 here.

The following media and solutions were used for the cell culture and for the experiments:

- Dulbecco's MEM (BIOCHROM AG, Leonorenstr. 2-6, D-12247 Berlin, Germany) with 10% (v/v) fetal calves' serum (FCS) and 350 µg/ml L-glutamine, called "Medium" hereinbelow,

- Dulbecco's MEM without FDS, called "serum-free Medium" (SFM) hereinbelow,

- Phosphate-buffered salt solution consisting of 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \bullet 7\ H_2O$, and 1.4 mM KH_2PO_4, pH 7.3, sterile-filtered, called "PBS" hereinbelow.

The cells were cultured in 10 ml of Medium at 37 °C in 75-cm^2 cell culture flasks (Corning B.V, Life Sciences, Koolhoveniaan 12, 1119 NE Schipol-Rijk, Netherlands) in a 5% CO_2 atmosphere.

Transfection experiments:

Transfection experiments were carried out with β-Gal⊕HuH-7 cells, human cervical carcinoma cells (cell line HELA-S3, available from the DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany, Number ACC 161), human urinary bladder carcinoma cells (cell line T-24, available from the DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, Number ACC 376), and cells of a cell line cultured from a human pancreatic carcinoma. The activity of the reporter gene β-galactosidase in the β-Gal⊕HuH-7 cells was determined by bioluminescence assay and this permitted a conclusion about the inhibition efficiency of the siRNA.

Transfections occurred 24 hours after seeding the cells in 96-well plates (number of cells on the date of seeding: $2 \bullet 10^4$ per well) and 12-well plates (number of cells on the date of seeding: 1-$2 \bullet 10^5$ cells per well). The transfection of the cells was accomplished either by means of FuGENE 6 Transfection Reagent (Roche Diagnostics GmbH) or with no transfection aid.

Transfection of β-Gal⊕Huh-7 cells with FuGENE 6:

Transfections were carried out in 96-well plates (quintuple determination) with a siRNA concentration of 50 nM per well. The transfection batches, which were pipetted into the cells, had a volume of 100 µl/5 wells. The transfection batches consisted of an amount of siRNA corresponding to the concentration to be tested, 3 µl of FuGENE 6 per 1 µg of RNA and SFM, with which they were made up to 100 µl. When performing the transfection, the siRNA was first placed in Eppendorf test tubes, mixed with SFM, and FuGENE 6 was then added. This mixture was incubated for 20 min at room temperature, then pipetted into the wells, and the cells were incubated for 48 hours at 37 °C and with a 5% CO_2 atmosphere.

Transfection of β-Gal⊕Huh-7 cells, cervical carcinoma cells, urinary bladder carcinoma cells, and pancreatic carcinoma cells, without transfection aid:

Transfections were carried out in 12-well plates in 3-fold batches. A concentration of 100 nM siRNA per well was used. For a transfection batch, 6 µl of a 20 µM siRNA was mixed with 1194 µl of SFM. The Medium was then removed from the cells and replaced by 400 µl of the transfection batch per well. After incubation at 37 °C with 5% CO_2 for 2½ hours, the transfection batch wise pipetted out, replaced by 1 ml of Medium, and the cells were incubated for 24 hours longer.

Cell lysis and bioluminescence assay:

Cell lysis was performed 24 hours or 48 hours after transfection, in the 96- or 12-well plates. To do this, the Medium was removed completely and the cells were washed twice with 100-µl or 500-µl portions of PBS, respectively. They were then covered at room temperature for 10 min with 50 µl or 250 µl of lysis solution (0.5 mM 1,4-dithiothreitol (DTT) and protease inhibitor (Complete Protease Inhibitor Cocktail Tablets 50x, Roche Diagnostics GmbH, Order No. 1 697 498) at the concentration in lysis solution recommended by the manufacturer (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 USA, Order No. ABX070LM), and finally thoroughly resuspended with a pipette, and transferred to Eppendorf test tubes on ice. They were then vortexed for 10-15 sec and centrifuged for 2 min at 16100 g. The supernatant was either used immediately for the assay or stored at –80 °C.

β-Galactosidase activity was measured in 5-ml hemolysis tubes (Sarstedt AG & Co., PO Box 1220, D-51582 Numbrecht, Germany, Order No. 55.476) in a Sirius Luminometer (Berthold Detection Systems GmbH, Bleichstr. 56-68, D-75173 Pforzheim, Germany). To measure the β-galactosidase activity, 5 µl of cell lysate was mixed with 30 µl of β-Gal assay buffer (100 nM Na phosphate, pH 8.0, 10 nM $MgCl_2$, 12.5 µg/ml Galacton [Applied Biosystems, Order No. GC020]) in a hemolysis tube and incubated for 1 hour at room temperature. 100 µl of β-Gal stop

mix (200 mM NaOH, 0.06% Emerald Enhancer II [Applied Biosystems, Order No. LAY250]) was then added and mixed briefly, and the luminescence was measured immediately on the luminometer.

siRNA isolation from cells:

24 hours after transfection in a 12-well plate, the total RNA and the siRNA were isolated from the cell lysates using the RNeasy Mini Kit (QIAGEN GmbH, Max-Volmer-Strasse 4, 40724 Hilden, Germany). The isolation was carried out according to the protocol "Isolation of total RNA from animal cells" of the QIAGEN company with the following modification:
After the first centrifugation step, the column effluent was stored at –80 °C for further use, since the short siRNA was located in it.

Detection of siRNA by Northern Blot:

Since the concentration of siRNA in the effluent could not be determined directly, it was determined in relation to the concentration of total RNA. The same amounts of siRNA from the effluents of the individual samples were used in each case. Volumes of effluent corresponding to identical amounts of siRNA were made up to identical volumes with 1:1 RLT buffer (from the RNeasy Mini-Kit)/70% ethanol, and 5 µl of E. coli tRNA (Roche Diagnostics GmbH, Order No. 109541) (10 µg/ml) was added. The siRNA was precipitated by adding 1/10 volume of 3 M sodium acetate (NaOAc), pH 5.4, and 3 volumes of absolute ethanol (EtOH) for 16 hours at -20 °C. The siRNA was pelletized by centrifuging for 10 min at 16100 g, the pellets were washed with 700 µl of 70% EtOH, and were centrifuged again for 5 min with the same g factor. After removal of the supernatant, the resulting siRNA samples were resuspended in 10 µl of stop buffer (95% (v/v) formamide, 0.1% (w/v) xylenecyanol, 0.1% (w/v) Bromphenol Blue, and 10 mM disodium ethylenediaminetetraacetate), denatured by 5 min incubation at 95 °C, placed on ice, and applied to an 18% denaturing sequencer gel (10 x 10 x 0.8 cm, 14.5 ml of sequencer gel concentrate, 2 ml of buffer concentrate, 3.5 ml of sequencer gel diluent, each from the Rotiphorese DNA sequencer system A431.1 (Carl Roth GmbH & Co., Schoemperlenstr. 1-5, D-76185 Karlsruhe), 20 µl of *N,N, N',N'*-tetramethylethylenediamine (TEMED) (Carl Roth GmbH & Co., Order No. 2367.3), and 60 µl of 10% ammonium peroxodisulfate (Carl Roth & Co., Order No. 9592.3). The gel run occurred for 2-3 hours at 150 V with 1x Tris-boric acid-EDTA (TBE) (10.8 g TRIS, 5.5 g boric acid, 4 ml 0.5 M EDTA, pH 8.0) as running buffer. The RNA was then transferred by the semi-dry method in a Hoefer Semi-Dry Transfer Unit (Amersham Biosciences Europe GmbH, Munziger Str. 9, D-79111 Freiburg, Germany, Order No. 80-621-86) electrophoretically (2 hours at 100 mA) to a Hybond N^+-Membrane (Amersham Biosciences Europe GmbH, Order No. RPN203B). The membrane was then dried for 16 hours at room temperature and then baked for 3 hours at 80 °C.

Preparation of radioactively labeled probes:

The single-stranded RNAs Gal3s (sequence No. 3) and HCV11s (sequence No. 2) were used as probes for the detection of the siRNA, and were radioactively labeled for the purpose at their 5' ends by means of T4 polynucleotide kinase (PNK). To this end, 3 µl of RNA (20 µM) was treated with 5 µl of γ-[^{32}P]-ATP (10 µCi/µl), 35 µl of H_2O, 5 µl of PNK 10x buffer, and 1 µl of PNK (10 U/µl) (PNK 10x buffer and PNK from New England Biolabs, Brüningstr. 50, Geb. G810, D-65926 Frankfurt am Main, Germany, Order No. M0201S), and incubated for 1 hour at 37 °C. The reaction mixture was then made up to 100 µl with H_2O and the probes were purified by means of a MicroSpin™-25 column (Amersham Biosciences Europe GmbH). The reaction mixture was then applied to the briefly vortexed columns that had been dry-centrifuged for 1 min at 0.7 g, and the probe was eluted by centrifuging again for 1 min at 0.7 g.

Hybridization: The ExpressHyb™ Hybridization Solution (Biosciences Clontech, Tullastr. 4, D-69126 Heidelberg, Germany) was used for the hybridization reaction. First, the membrane was moistened briefly with the siRNA in H_2O and prehybridized on a shaker for 30 min at 37 °C with 5 ml of ExpressHyb Hybridization Solution. The ExpressHyb Hybridization Solution was then replaced by a mixture of 5 ml of ExpressHyb Hybridization Solution and 250 ng of the radioactively labeled probe and the blot was hybridized for 1 h at 37 °C. This was followed by several washing steps at room temperature, first with wash solution 1 (0.3 M NaCl, 30 mM sodium citrate, pH 7.0, 0.05% sodium dodecyl sulfate SDS)), which was replaced three times over a period of 45 min, and then 45 min with wash solution 2 (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, 0.1% SDS), replacing the solution twice. For development, the membrane was placed on filter paper, wound around with plastic film, and exposed for 16 h in a cartridge to a storage phosphor screen (Amersham Biosciences Europe GmbH). This was finally read using a Storm 820 phosphorimager (Amersham Biosciences Europe GmbH, Order No. 63-0035-52).

Results:

All experiments for determining β-galactosidase activity were carried out 5 times and the measured results were averaged. The results of the changes of β-galactosidase activity caused by the transfections are shown graphically in Fig. 3. The average of untreated cells was defined as 1.0. The values determined for transfected cells are shown in relation thereto.

It was found that the gene expression could be reduced by the modified siRNAs by as much as 56% in the case of GalChol-s.

The result of the transfection experiment performed with β-Gal⊕Huh-7 cells without a transfection aid is shown in Fig. 4. The inhibition of β-Gal expression was determined by measuring the β-galactosidase activity. The siRNA taken up by the cells was determined in the cell lysate by Northern Blot, by hybridization with the radioactively labeled single-stranded RNA probes Gal3s and HCV11s.

The upper part of Fig. 4 shows in each case the β-Gal activity of the cells treated with the indicated siRNAs. The bottom part of the illustration shows in each case the Northern Blot of the siRNA isolated from these cells. From left to right, the first four tracks of the Northern Blot show the siRNA isolate of the cells treated with the modified siRNAs HCVChol-s, HCV32-s, GalChol-s, and GalC32-s. The following three tracks show siRNA isolates of the cells treated with the unmodified siRNAs HCV, Gal, and K22.

Fig. 4 shows that siRNAs with the modifications Chol-s and C32-s are taken up by cells even without adding transfection aids, and cause inhibition of the expression of a target gene. Unmodified siRNAs, on the other hand, are not taken up by cells and do not inhibit the expression of β-galactosidase.

Figures 5a, 5b, and 5c each show a Northern Blot from siRNA isolated from cells from pancreatic carcinoma cells (Fig. 5a), cervical carcinoma cells (Fig. 5b), and urinary bladder carcinoma cells (Fig. 5c). The tracks of each Northern Blot show, from left to right, siRNA isolates from cells that were treated with the following siRNAs with no transfection aid: HCVChol-s (Track 1), HCV32-s (Track 2), GalChol-s (Track 3), GalC32-s (Track 4), HCV (Track 5), and Gal (Track 6). Figures 5a, 5b, and 5c show that the dsRNA pursuant to the invention is also taken up without a transfection aid by cells other than liver cells.

Patent Claims

1. Double-stranded ribonucleic acid (dsRNA) with a strand S1 complementary at least in sections to a target gene, characterized by the fact that at least one lipophilic group is bonded to the dsRNA.

2. dsRNA pursuant to Claim 1, characterized by the fact that the lipophilic group is bonded only to the strand S1 or only to another strand S2 at least largely complementary to strand S1 of the dsRNA.

3. dsRNA pursuant to Claim 1 or 2, characterized by the fact that the lipophilic group is bonded, in particular covalently, to the 5' end of the strand S1 and/or of the strand S2.

4. dsRNA pursuant to one of the preceding claims, characterized by the fact that the lipophilic group is bonded exclusively to the 5' end of the strand S2.

5. dsRNA pursuant to one of the preceding claims, characterized by the fact that the lipophilic group has the structure of a steroid, particularly of a sterol, preferably of cholesterol or of an aliphatic hydrocarbon, particularly a branched one.

6. dsRNA pursuant to one of the preceding claims, characterized by the fact that the lipophilic group has the structure of (6-hydroxyhexyl)carbamic acid cholesteryl ester or of 12-hydroxydodecanoic acid bis(decyl)amide.

7. dsRNA pursuant to one of the preceding claims, characterized by the fact that the dsRNA, at its end that has the 3' end of the strand S1, has a single-stranded overhang formed of 1 to 4, especially 1 or 2, nucleotides on the strand S1.

8. dsRNA pursuant to one of the preceding claims, characterized by the fact that the strand S1 preferably has between 16 and 30, especially between 16 and 25, and most preferably between 20 and 25 nucleotides.

9. dsRNA pursuant to one of the preceding claims, characterized by the fact that the target gene is a target gene expressed, particularly specifically, in liver cells, pancreatic cells, cervical cells, or cells of the urinary bladder.

10. dsRNA pursuant to one of the preceding claims, characterized by the fact that the target gene originates from the HCV genome, particularly the untranslated region located at the 3' end (3'-UTR) of the HCV gene.

11. Method for preparing a dsRNA pursuant to one of the claims 1 to 10, characterized by the fact that the RNA constituting the strand S1 and/or the strand S2 is prepared by solid phase synthesis in the direction from the 3' end to the 5' end, and a lipophilic molecule (10, 17) that has a phosphoramidite group is coupled in the last step of the synthesis to the 5' end of the RNA.

12. Method pursuant to Claim 11, characterized by the fact that the synthesis of the lipophilic molecule (10, 17) that has the phosphoramidite group comprises the phosphitylation of a free OH group to give the phosphoramidite group.

13. Method pursuant to Claim 11 or 12, characterized by the fact that the synthesis of the lipophilic molecule (10, 17) that has the phosphoramidite group comprises conversion of cholesteryl chloroformate (8) to an acid amide (9).

14. Method pursuant to Claim 11 or 12, characterized by the fact that the synthesis of the lipophilic molecule (10, 17) that has the phosphoramidite group comprises the reaction of 12-hydroxylauric acid (11) with di-n-decylamine (15) to form an acid amide bond.

15. Method pursuant to one of the claims 11 to 14, characterized by the fact that the lipophilic molecule (10, 17) that has the phosphoramidite group is [6-(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]carbamic acid cholesteryl ester (10) or 12-[(2-cyanoethoxy)-N,N-diisopropylaminophosphanyloxy]dodecanoic acid bis(decyl)amide (17).

16. Use of a dsRNA pursuant to one of the claims 1 to 10 to inhibit the expression of a target gene contained in cells.

17. Use pursuant to Claim 16, characterized by the fact that no agent is used by which uptake of dsRNA in the cells is brought about.

18. Use pursuant to Claim 16 or 17, characterized by the fact that the expression of the target gene in liver cells, cells of the pancreas, of the uterus, or of the urinary bladder, particularly those infected with the HCV virus, is inhibited.

19. Use pursuant to one of the claims 16 to 18, characterized by the fact that the target gene originates from the HCV genome, particularly the untranslated region located at the 3' end (3'-UTR) of the HCV genome.

20. Drug for inhibiting the expression of a target gene contained in cells, characterized by the fact that a dsRNA pursuant to one of the claims 1 to 10 is contained in the drug.

21. Drug pursuant to Claim 20, characterized by the fact that it contains no agent by which the uptake of dsRNA in the cells is brought about.

22. Drug pursuant to Claim 20 or 21, characterized by the fact that the cells are liver cells or cells of the pancreas, of the uterus, or of the urinary bladder.

23. Drug pursuant to one of the claims 20 to 22, characterized by the fact that the target gene originates from the HCV genome, particularly the untranslated region located at the 3' end (3'-UTR) of the HCV genome.

SEQUENCE PROTOCOL

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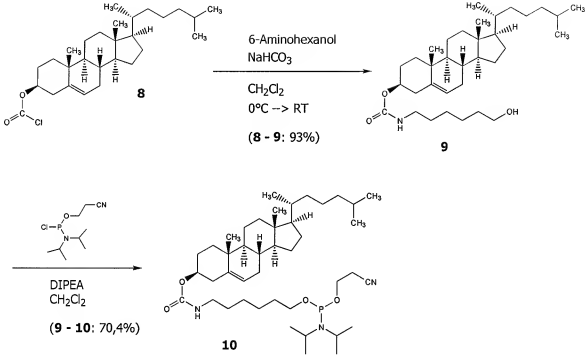
Abstract

The invention relates to a double-stranded ribonucleic acid with a strand S1 complementary at least in sections to a target gene, wherein at least one lipophilic group is bonded to the dsRNA.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(8 - 9: 93%)
9
DIPEA
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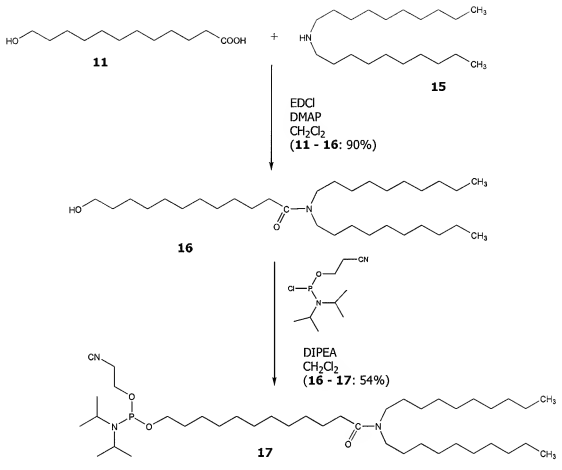
(9 - 10: 70,4%)
10

Fig. 1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COOH
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+
HN
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CH3
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DMAP
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(11 - 16: 90%)
HO
C
N
O
CH3
CH3
16
CN
O
Cl—P
N
DIPEA
CH2Cl2
(16 - 17: 54%)
CN
O
P
N
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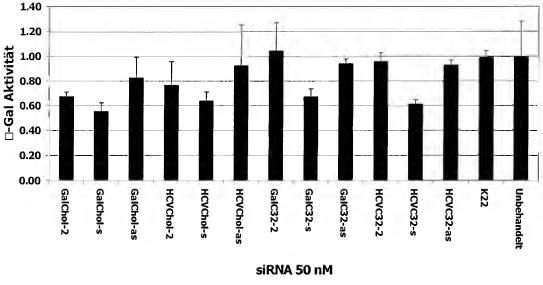
O
CH3
CH3
17

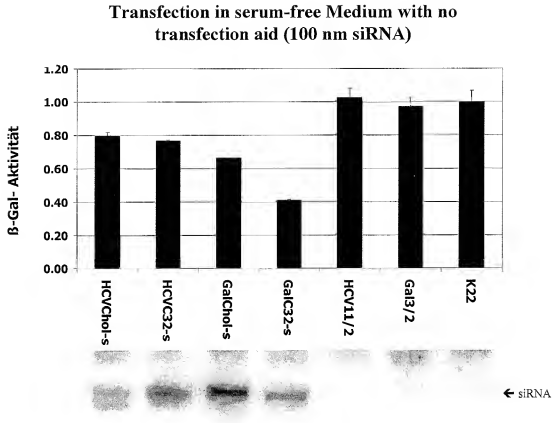
Fig. 2

RNAi: Transfection of the modified SIRPLEX molecule in β-Gal⊕HUH-7 cells



Ordinate: β-Gal Activity
Unbehandelt = Untreated

Fig. 3



Ordinate: β-Gal activity

Fig. 4

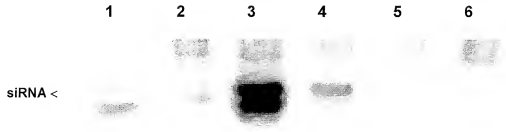


Fig. 5 a

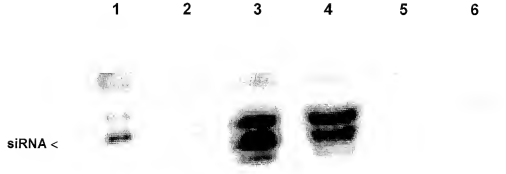


Fig. 5 b

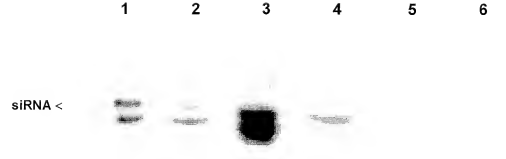


Fig. 5 c